Plant Physiol. (1989) 91, 338–344
0032-0889/89/91/0338/07/$01.00/0

Received for publication September 20, 1988
and in revised form April 28, 1989

Distribution of Endogenous Indole-3-Acetic Acid and Compression Wood Formation in Reoriented Branches of Douglas-Fir[1]

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ABSTRACT

Five-year-old segments of intact 7-year-old branches of Douglas-fir (*Pseudotsuga meziesii* [Mirb.] Franco) were reoriented to determine the relation between indole-3-acetic acid (IAA) and the formation of compression wood. Eight branches per treatment were either left at their original angle (mean of 69°, the control), or bent proximal to the segment to reorient it up or down 30°. Differentiating xylem tissue from the upper and lower sides of each segment was collected and extracted separately for IAA analysis by in-line fluorescence detection of free IAA and IAA methyl ester after sequential C_{18} reversed-phase high performance liquid chromatography. The IAA methyl ester was confirmed by gas chromatography-mass spectroscopy. Compression wood formed on the upper side of branches reoriented up and on the lower side of controls or branches reoriented down. IAA was present in all samples. The difference in IAA concentration between upper and lower sides was either not correlated, or negatively correlated in segments reoriented down, with both the occurrence of compression wood and the rate of new tracheid production. Mean concentrations for whole branch segments were not affected by the treatments, regardless of whether IAA concentrations were expressed on a surface area, weight, or cell basis.

Douglas-fir branches, like other conifer branches, are plagiotropic. They normally grow at a equilibrium position and, if bent vertically or horizontally out of this position, form CW[2] where its action will bend the branch back to the equilibrium position (19). Thus, if a branch is bent down it forms CW on the underside, and if bent up it forms CW on the upper side. In Douglas-fir this plagiotropic habit may be retained in rooted branch cuttings (15, 16).

A common hypothesis, with little evidence to support it (19), explaining the location of CW formation is that when a branch is moved out of its equilibrium position, IAA is transported to the side of the branch farthest away from the equilibrium position, and the high concentration of IAA on that side stimulates CW formation (18). This hypothesis is a modification of the Cholodny-Went model for differential cell elongation during gravitropism in herbaceous plants, a model which is being questioned (4, 9). CW formation generally involves both the rate of cell production and changes in cell wall differentiation rather than cell elongation. Annual rings are usually, but not necessarily, wider on the side of the branch with CW. Compared to normal tracheids, CW tracheids are round in cross section and have intercellular spaces and thick lignified walls. In contrast to geotropic curvatures which result from differential cell elongation, the mechanical action of CW apparently develops during secondary wall formation (21).

With the development of analytic chemistry techniques for measuring plant growth regulators, many hypotheses about the regulation of cambial activity by IAA are being reassessed (6, 14). For example, seasonal changes in IAA concentration measured by gas chromatography-selected ion monitoring are not enough to explain either seasonal changes in cambial activity or the differentiation of cambial derivatives (13).

The strongest evidence for the role of IAA in CW formation is that high concentrations of exogenous IAA stimulate CW formation in vertical conifer stems that would not otherwise form CW (19). There are, however, no definitive measurements of IAA in relation to CW formation or on upper and lower sides of branches (6). Measurements of extractable and diffusible IAA made with the *Avena* curvature test (8) or the *Triticum* section test (7) have shown higher levels of promoters, interpreted as IAA, on the lower side of branches or tilted conifer stems. Starbuck and Roberts (15, 16) were not able to demonstrate lateral transport of exogenously applied [^{14}C]IAA in plagiotropic cuttings from Douglas-fir, but they did show that the [^{14}C]IAA accumulated at the site where CW formed on the adaxial side of rooted cuttings. Similarly, IAA applied to either the upper or lower side of tilted, decapitated Douglas-fir stems could stimulate growth on the lower side (20). Mechanical stress has, for many years, been suggested as the stimulus for CW formation (19). Ethylene is produced by pine stems that are severely bent (2). Savidge *et al.* (13) found aminocyclopropane-1-carboxylic acid, a precursor of ethylene, associated with CW formation in lodgepole pine (*Pinus contorta* Dougl. ex Loud.) branches. Blake *et al.* (1) investigated the relation of ethylene to hyponasty, or upward bend-

[1] Paper 2427 of the Forest Research Laboratory, Oregon State University, Corvallis, OR 97331. Mention of trade names or commercial products does not constitute recommendation for use by the authors or their institutions.

[2] Abbreviations: CW, compression wood; TEAA, triethylamine ammonium acetate; MeIAA, methyl ester of IAA.

ing (they did not look for CW), in branches of young Arizona cypress (*Cupressus arizonica* Greene). They found that cutting the stem above the branch increased both hyponasty and ethylene production on the lower side of the branch. Gassing branches with ethylene also increased hyponasty.

The experiment in this paper was designed to test the hypothesis that high IAA concentrations, measured with analytical chemistry techniques, are associated with CW formation in Douglas-fir branches moved out of their equilibrium position. IAA was extracted from differentiating xylem of the upper and lower sides of branch segments that had been reoriented without bending the segment itself to reduce mechanical bending effects. Measurements were taken early in the growing season to look at early stages of CW formation with relatively little new tissue. Only differentiating xylem tissue, the tissue that forms CW, was investigated so that the results would not be confounded by IAA in the phloem. For example, early in the growing season, lodgepole pine has about 5 times more IAA in the recently formed xylem than in the phloem and cambial zone (12). All the differentiating xylem tissue was sampled because CW formation requires gravitational stimulus during the entire differentiation process (5), and thus should also require high IAA concentrations during the whole process.

MATERIALS AND METHODS

Field Sampling

All branches were on trees on the east and west edges of a 17-year-old Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) plantation at Peavy Arboretum near Corvallis, Or. Twenty-four, 7-year-old, vigorous branches of comparable size were selected in January 1988. All branches were 2.4 to 3.0 m long and had elongated 23 to 30 cm the previous summer. Eight replicates of the three reorientation treatments (up, control, and down) were assigned randomly among the 24 branches.

We investigated the 5-year-old segment of the main axis of each branch. These segments were needle-free and were large enough (25 cm long and 2 cm in diameter) to provide enough uniform tissue for analysis. All small branches were removed from each segment. On January 22, more than 3 months before budbreak, the angle to vertical of the midpoint of each 5-year-old segment was measured with an inclinometer.

The reorientation method was designed to avoid girdling or wounding the main axis of the branch and to reduce bending stresses induced within the segment (Fig. 1). For each branch, plastic-covered wire was tied around the base of the lateral branches at the distal end of the 6-year-old segment. The wire was then pulled tight and attached to the base of branches above (for reorientation up and for controls) or below (for reorientation down) the treated branch. Segments were reoriented 30° up or down from their original angle (mean of 69°); controls were restrained at their original angles. Although the segments were not bent when reoriented, the bending moment on the reoriented segments did change, thereby also changing compressive and tensile stresses, because the horizontal distance to the center of gravity changed (22). Assuming an initial angle of 69°, rotating up 30° decreases the bending moment about 30% and rotating down 30° increases it about 6%. The angles were checked at 2- to 3-week intervals throughout the experiment to assure that they remained constant. A few branches were readjusted initially, but then the angle stayed within 2°.

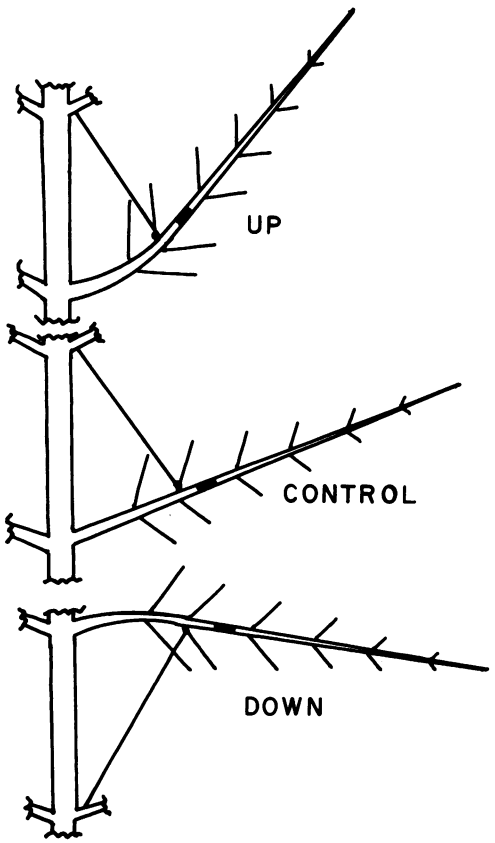

Figure 1. Diagrammatic representation of reoriented branches. Each annual segment is delimited by lateral branches. The branches are reoriented up or down 30° or held at their original angle (control) by plastic-covered wires attached to lateral branches proximal to the sample segment. The 15-cm segment from which new xylem tissue was taken for auxin analysis is marked in black.

Tissue samples were taken on May 4 and 5, 1988, 3 to 10 days before budbreak on comparable branches of the same trees. The leaves had elongated within the buds so that buds were 1.4 to 2.8 cm long, and both cambial activity and CW formation had started. While each sample branch was still held in place, a segment 15 cm long was marked on the bark in the center of the 5-year-old segment, and the upper and lower halves of each 15-cm segment were separated by a knife cut into the wood. The 15-cm segment was then sawn off. Cross-sectional disks about 1.5 cm thick were cut just distal and proximal to the 15-cm segment and were put in fixative (formalin, glacial acetic acid, and 50% ethyl alcohol in water, 1:1:45 v/v/v) for later anatomical study.

New (1988) xylem tissue was scraped from the 15-cm segments in the field. The upper and then the lower halves were sampled separately. First the bark was removed. Subsequent anatomical observations showed that, upon removal, the bark tore through enlarging cells just on the xylem side of

the cambial zone, as illustrated by Savidge *et al.* (12). The exposed xylem surface was then scraped using stainless steel spoons with the tip ground to a concave edge of about the same curvature as the surface of the branch. This scraping removed most of the new xylem. The amount of tissue removed was subsequently confirmed from sections of the scraped segments. The sample tissue was put directly into test tubes containing 5 mL methanol at 0°C, 2 mL of 20 mM ammonium acetate buffer at pH 6.5, and the following antioxidants: 30 mg sodium diethyldithiocarbamate; 20 μL butylated hydroxytoluene; 20 mg·mL^{-1} in methanol; and 100 μL mercaptoethanol. The tissue was exposed to the air for <1 min. The surface area of the samples (24.3–63.1 cm^2) was calculated from measurements of the length and width of the bark section removed from each sample. Fresh weights of the samples (0.42–2.12 g) were determined by weighing the test tubes and contents before and after adding the scrapings; dry weights were not determined.

Laboratory Analysis

An internal standard of approximately 40,000 dpm [^{3}H] IAA (Amersham, 30 Ci/mM) was added to each sample tube. The tissue in methanol and antioxidants was ground with a polytron (Brinkmann Instruments, Westbury, NY) and then centrifuged, and the supernatant collected. The pellet was resuspended in ammonium acetate buffer and centrifuged again, and the supernatant combined with the previous supernatant. The combined supernatant was passed through two columns in series, first polyvinylpolypyrrolidone (10 mL) and then Whatman DE-32 cellulose (10 mL). Both columns were washed with ammonium acetate buffer containing antioxidants. IAA was eluted from the DE-32 column onto a Sepralyte C_{18} column (2 mL) with 1 M acetic acid adjusted to pH 3.1 with NH_4OH, then from the C_{18} column with 4 mL methanol. The eluate was dried in a Speed Vac Concentrator (Brinkmann) and stored at −20°C until analysis.

IAA was separated from many contaminating substances with two sequential HPLC steps each using two Beckman model 112 pumps, with a model 480 controller fitted with a C_{18} reversed-phase column (Altex Ultrasphere-ODS, 250 × 4.6 mm) and coupled with a Perkin-Elmer 650-10S Fluorescence Spectrophotometer set at Ex_{285}/Em_{345} nm. The solvents were HPLC-grade methanol (Baker) and 20 mM TEAA buffer at pH 3.6 filtered through a C_{18} millipore system.

For the first HPLC step, the dry sample was dissolved in 50 μL methanol, and a 10-μL aliquot with 10 μL TEAA buffer was injected into the HPLC. The mobile phase was 45% methanol and 55% TEAA buffer; the flow rate was 1 ml·min^{-1}. The fraction of the eluate containing IAA (retention time of 7.5–9 min, Fig. 2) was collected and dried in the Speed Vac Concentrator. At this point, the recovery ranged from 35 to 83% (mean of 58%).

For the second HPLC step, the dried fraction described above was methylated with fresh diazomethane (3), dissolved in 40 μL methanol and water (1:1 v/v), injected into the HPLC (same column and detector as described above), and eluted with methanol:water (1:1 v/v) at 1 ml·min^{-1}. The MeIAA eluted at about 11.6 min and was resolved to the baseline (Fig. 2).

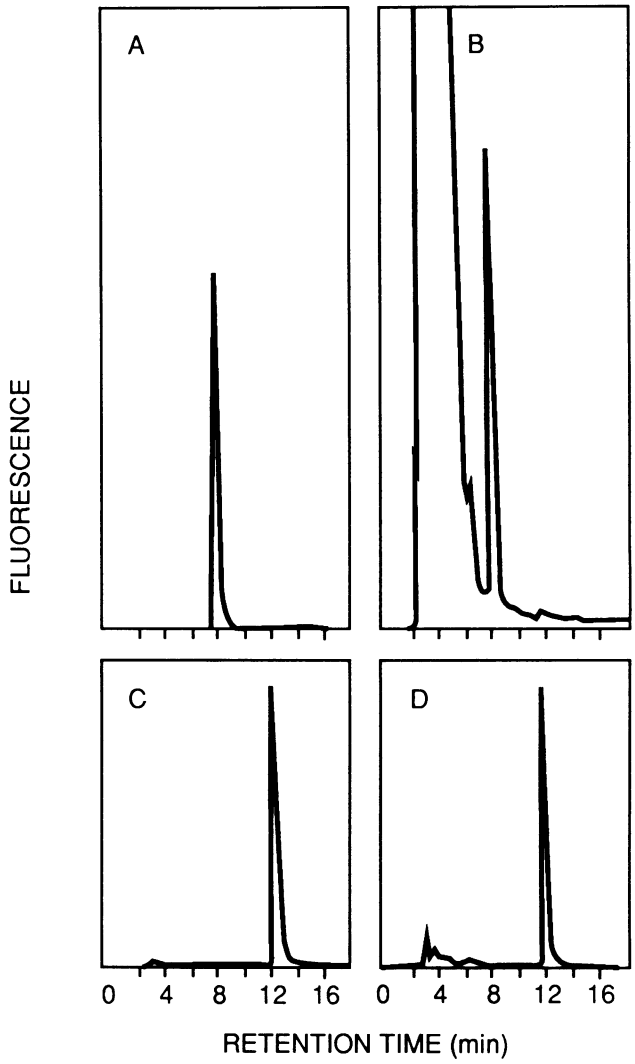

Figure 2. HPLC chromatograms of (A) an IAA standard, (B) sample containing IAA, (C) MeIAA standard, and (D) methylated sample.

The amount of IAA in the original sample was calculated using the recovery rate from the first HPLC step. The amount of MeIAA was calculated using peak areas and a standard curve from methylated authentic IAA. The MeIAA peak in one sample was confirmed by GC-MS (Fig. 3). The recovery rate between the first HPLC step and after the second HPLC step ranged from 67 to 100% (mean of 90%). Overall recovery rate ranged from 29 to 80% (mean of 52%). Although fluorescence detection does not preclude the presence of a second compound in the putative IAA peak, such contamination seems unlikely based on the baseline resolution of the putative IAA peak, GC-MS confirmation of IAA in that peak, and wavelength specific fluorescence detection employing excitation and emission wavelengths near the optima for IAA. A contaminant would have a to coelute in two HPLC steps and overlap significantly in both excitation and emission wavelengths.

Anatomical observations were made from microtome sections of fixed paraffin-embedded and unembedded tissue. All

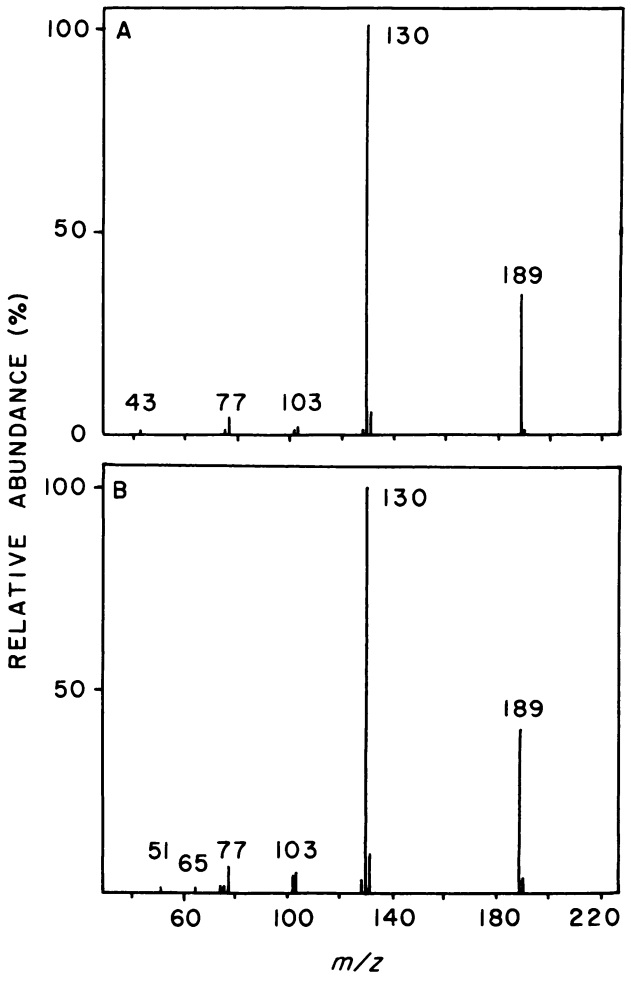

Figure 3. Mass spectra of (A) methylated authentic IAA and (B) MeIAA extracted from differentiating xylem of Douglas-fir branches.

sections were stained with toluidine blue. CW formation was classified as "weak" if there were rounded tracheids with intercellular spaces and as "strong" if there were both rounded cells with intercellular spaces and thickened cell walls (Fig. 4). The number of new cells (tracheids) per cm^2 of bark surface area was estimated by counting the number of new cells per radial file and then multiplying by the number of files per cm^2. Because enlarging tracheids were usually crushed during sawing and sectioning of the disks, the only consistently reliable measurement of new cells per radial file was for cells that had finished enlarging and had secondary walls stiff enough to resist crushing. The number of cells per file was the mean of counts from five radial files, determined for each sample. The number of radial files per cm^2 was set at a constant 2500 for all samples, based on measurements of tangential cell diameters from transverse sections (mean of 426 cells·cm^{-1} or a cell width of 23 μm) and cell lengths from tangential sections (mean of 5.9 cell·cm^{-1} or a cell length of 1.7 mm). Therefore, the only variable in cell number calculations was the radial number of new cells with secondary walls.

Statistical analyses used PC SAS (10) for analysis of variance (ANOVA) with Duncan's multiple range test, and *t*-tests.

RESULTS

Compression wood formed on the upper side of all eight branches reoriented up, on the lower side of all eight branches reoriented down, and on the lower side of six of the eight control branches. Half of the branches reoriented up formed only weak CW; but all the other branches that formed CW had strong CW with thick cell walls. CW formation was consistently correlated with a increased number of new cells per radial file; but because the cell number on the side opposite the CW was reduced, the mean number did not differ among treatments (Table I).

Reorienting branches down caused CW to form earlier than usual. The underside of branches usually forms a band of normal wood before producing CW. In the year before treatment, the growth ring on the underside of all 24 branches had a band of normal cells at least 8 cells wide and, on average, 19 cells wide. In the eight branches reoriented down, CW with round cells and thick walls was produced after a band of normal cells only two to four cells wide (Fig. 4).

Free IAA was found by both IAA and MeIAA fluorescence in all 24 samples (Table II). Mean concentrations for all three treatments, both upper and lower sides, were: 5.9 ng·cm^{-2} (range of 1.9–10.2 ng·cm^{-2}) on the basis of surface area; 299 ng·g^{-1} (range of 85–650 ng·g^{-1}) on the basis of fresh sample weight; 0.248 pg·$cell^{-1}$ (range of 0.024–0.640 pg·$cell^{-1}$) on the basis of new cells with secondary walls. IAA concentrations were not significantly different for the upper and lower sides in branches reoriented up, despite the fact that CW formed on the upper side (Table II). In branches reoriented down, and in control branches on a weight and cell basis, there was more IAA on the upper side although the CW formed on the lower side. Although there was no difference between upper and lower sides in branches reoriented up, there was relatively more IAA on the upper side of branches reoriented up than in control branches or those reoriented down on a weight or cell basis (Table II).

The results were similar whether IAA concentration was expressed on a surface area, weight, or cell basis, but the treatments accounted for the greatest amount of variability (R^2 = 0.70) in the differences in IAA concentration when expressed on a cell basis (Table II). The difference in IAA between the upper and lower sides of branches reoriented down was 194% of the mean on a cell basis, but only 73 or 87% of the mean on an area or weight basis.

Despite significant differences in IAA concentration between upper and lower sides in control branches and branches reoriented down (no significant difference in branches reoriented up), there were no differences among treatments for mean IAA concentration of whole branches (Table II). Thus, reductions in IAA on the side with CW were balanced by increases on the opposite side. This balancing could have resulted from lateral IAA transport or from changes in the rate of IAA production or destruction.

If CW formation destroys IAA, the amount of IAA per unit surface area should decrease as the radial number of CW cells increases. A regression predicting IAA per cm^2 for the 22 samples that had CW showed a significant ($p = 0.042$) decrease in IAA with increase in cell number (IAA ng·cm^{-2} =

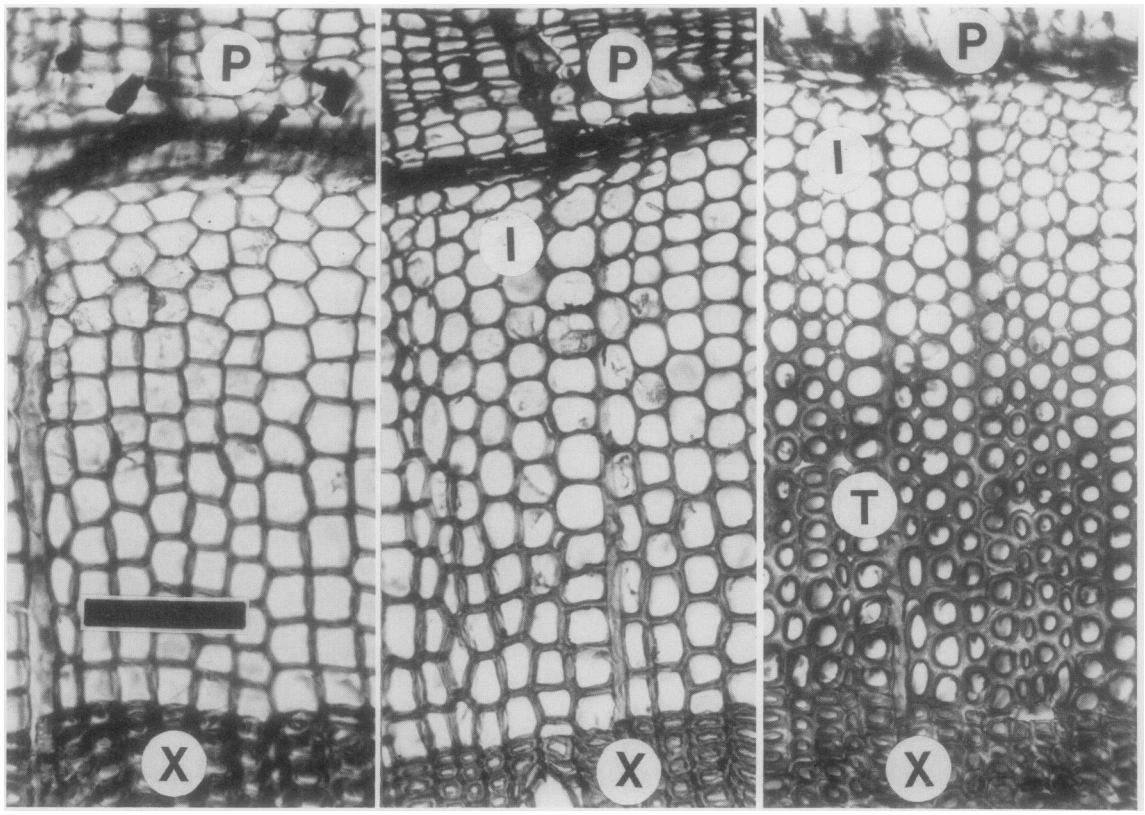

Figure 4. Photomicrographs of transverse sections showing new normal wood on the left, weak CW in the center, and strong CW on the right. P = phloem, X = last year's xylem, I = CW with intercellular spaces, and T = CW with thick walls. The bar in normal wood is 0.1 mm long.

Table I. *Effect of Reorientation on Radial Numbers (Mean ± SE) of New Cells with Secondary Walls; n = 8 for All Treatments*

Difference in cell number is the number on the lower side of the branch subtracted from the number on the upper side (negative numbers indicate more cells on the lower side); mean cell number is the mean of the two sides.

Treatment	Difference in Cell Number*	Mean Cell Number*
Up	7.5a ± 1.5	14.8a ± 0.8
Control	−8.5b ± 1.6	11.9a ± 1.1
Down	−12.0b ± 0.9	11.5a ± 1.3

* Within a column, values followed by the same letter are not significantly different from each other (Duncan's multiple range test, $p = 0.01$). All values are significantly different from zero (t test, $p < 0.001$).

8.59 − 0.21 CW radial cell number), but the regression accounted for little of the variability in IAA ($R^2 = 0.19$). Therefore, the number of CW cells is a relatively unimportant variable in determining the amount of IAA.

DISCUSSION

IAA was present in all our samples, measured just before budbreak. The amounts we measured (85–650 $ng \cdot g^{-1}$ fresh weight) were similar to the 96 and 722 $ng \cdot g^{-1}$ fresh weight measured for dormant pine branches (13), but considerably less than the 2000 $ng \cdot g^{-1}$ measured in pine stems at a comparable developmental stage. The maximum of 10.2 $ng \cdot cm^{-2}$ of surface area was much less than the 200 $ng \cdot cm^{-2}$ measured for pine stems in June (17). Thus, IAA concentrations seem to be much lower in branches than in stems, despite the fact that nearly all leaves and buds, the presumed producers of IAA, are on branches of larger trees. If further research supports the finding of lower IAA concentrations in branches than in stems, then the question would be: where does the IAA in stems come from? It it produced by stem cambium itself? This issue must be resolved by further study.

The hypothesis that CW forms on the side of a branch where IAA concentration is high must be rejected. In branches reoriented up, CW formed when there was no difference in extractable IAA concentration between the upper and lower sides. In branches reoriented down, CW formed on the side with the lower IAA concentration. We found more than twice as much extractable IAA per cm^2, and more than 5 times as much per cell, on the upper side as on the lower side of branches reoriented down. There is a comparable negative correlation between IAA concentration and growth in gravistimulated soybean (*Glycine max*) hypocotyls (9). If IAA concentration is negatively correlated to CW formation in branches, then positive correlations measured in tilted stems may be spurious. One would think that any hypothesis explaining CW formation should apply both to stems and to branches.

We measured total extractable IAA, but CW formation may be better related to diffusible IAA or to IAA plus IAA

Table II. *Effect of Reorientation Treatments on Auxin Concentrations; n = 8 for All Treatments*

Differences in IAA are between the upper and lower sides (negative values indicate a higher concentration on the lower side); means are for the whole branch sample. IAA concentrations are expressed on the basis of bark surface area or of fresh weight of sample, or per number of new cells (tracheids) with secondary wall.

Treatment	Difference in IAA*			Mean IAA*		
	Area	Weight	Cell	Area	Weight	Cell
	$ng \cdot cm^{-2}$	$ng \cdot g^{-1}$	$pg \cdot cell^{-1}$	$ng \cdot cm^{-2}$	$ng \cdot g^{-1}$	$pg \cdot cell^{-1}$
Up	−0.8a,ns	−27a,ns	−0.14a,ns	5.9a	305a	0.21a
Control	1.2a,ns	139b	0.23b	6.0a	282a	0.23a
Down	4.2b	260b	0.33b	5.7a	301a	0.17a

* Within a column, values followed by the same letter are not significnatly different from each other (Duncan's multiple range test, $p = 0.01$). Only those values followed by ns are not significantly different from zero (t-test, $p > 0.01$).

conjugates. Using the *Avena* curvature bioassay, Onaka (8) measured 2 to 4 times as much diffusible auxin on the lower side of inclined pine stems that formed CW. Recent measurements show that IAA measured in diffusate from pine cambium was the same whether using *Avena* curvature bioassay or analytical chemistry techniques (23). Diffusable IAA from reoriented branches has not been measured. Wodzicki *et al.* (23) have identified a putative IAA conjugate in cambial tissue diffusate in vertical pine stems.

The hypothesis that differences in IAA concentrations occur from lateral transport cannot be rejected on the basis of our results. Despite large differences (up to 10 times on a cell basis) in concentration between upper and lower sides of branches reoriented down, mean IAA concentration for whole branches was the same in all treatments. Thus, the upper side gained enough IAA to compensate for the decrease in IAA on the lower side, and this compensation could have resulted from lateral IAA transport. If lateral transport indeed occurred, the direction was toward the equilibrium position and away from both the expected direction and the direction of transport observed using exogenously applied [^{14}C]IAA (15, 20).

In branches reoriented down, IAA concentration was negatively correlated with both the rate of cell production, as measured by new cells per radial file, and the process of CW differentiation. Although in other systems the processes of cell production and CW differentiation appear to be controlled separately and are not always positively correlated (19), in our branches neither process appears to be promoted by high IAA concentrations.

If CW formation uses up IAA, there should be less IAA on the upper side of branches reoriented up, but in fact there was no significant difference in concentration. Overall, IAA concentration significantly decreased as the number of CW cells increased, but the decrease was small and accounted for little of the variation in IAA. Therefore, auxin loss does not appear to be a viable alternative hypothesis.

It seems most likely that, as in other aspects of cambial activity (6, 11, 14), IAA is only one of several factors required for CW formation. IAA is present on both sides of branches whether CW forms or not, so mere presence is not enough. CW forms on the upper side of branches reoriented up even when there is no difference in concentration. Ethylene is likely to be the significant factor in CW formation (1). An ethylene precursor is present only on the lower side of lodgepole pine branches, the side that forms CW (13). Although ethylene may be important for CW formation, our results suggest that it is not produced because of high free IAA levels, in that IAA concentration and CW formation can be negatively correlated. Necesanỳ (7) extracted new xylem tissue from upper and lower sides of pine branches and tested the extracts with paper chromatography and the *Triticum* section bioassay. He found growth promoters generally on the lower side (most at the same R_F as authentic IAA) and more inhibitors, "or inhibitory levels," on the upper side.

Although IAA was present and may be required for CW formation, the concentration of endogenous IAA was not positively related to CW formation. IAA was present on both sides of branches and is present throughout the year in dormant and active cambium of stems (14, 17). Thus, although IAA is always present whether or not CW forms, some other substance may regulate CW formation. If endogenous IAA is not the stimulus for CW formation, then why does exogenous IAA induce CW? Perhaps differences in tissue sensitivity to IAA, rather than IAA concentration, modulate CW formation (9). Perhaps the concentrations required to induce CW formation are so high that they have traumatic effects, inducing ethylene or some other factor that causes CW to form. In any case, the role of IAA in CW formation, as in other aspects of cambial activity, needs to be reexamined.

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